STUDIES ON THE GROWTH OF INTEGUMENTARY PIGMENT IN THE LOWER VERTEBRATES

I. The Origin of Artificially Developed Melanophores on the Normally Unpigmented Ventral Surface of the Summer Flounder (Paralichthys dentatus) [1]

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Considerable evidence has accumulated to indicate that melanophores may be grown experimentally on certain fishes and amphibians in areas where these cells fail to develop naturally. Cunningham (1891, 1893, and 1895), working with several species of flatfishes; von Frisch (1911), using *Esox* and *Nemadulus;* and Osborn (1940*a, b*, and *c*), studying the summer flounder (*Paralichthys dentatus*) and the common bullhead (*Ameiurus melas*) have all reported success in growing melanophores on the normally unpigmented ventral [2] surfaces of these teleosts. Experimenting with the urodele, *Salamandra maculosa,* Herbst and Ascher (1927) were able to develop abnormal amounts of pigment ventrally. In spite of these observations the origin of the newly developed melanophores has remained an open question. Alternative possibilities are obvious: either they differentiate *in situ* or they migrate in from other areas.

This paper brings together the results of experiments referred to in an earlier report (Osborn, 1940*a*) designed to gain more information concerning the source of experimentally developed melanophores.

Materials and Methods

A freshly caught stock of adult flounders 15 to 18 inches long was maintained to avoid abnormal conditions in pigmentation which sometimes arise from prolonged sojourns in unnatural laboratory surroundings. The desired amount of pigmentation was developed ventrally in

[1] Contribution No. 296 of the Woods Hole Oceanographic Institution, whose research facilities and financial aid provided for this investigation are genuinely appreciated.

[2] The term "ventral" will be used to refer to the lower normally unpigmented surface of the animal. In the flatfishes the unpigmented side is more strictly the right or the left side, depending upon the species. In the summer flounder the right side is white.

an apparatus similar to that previously described (Osborn, 1940*a*) with minor improvements. When a fish was sacrificed, at least 40 scales plucked from widely separated areas on the ventral surface were fixed in 5 per cent neutral formalin. Of these, ten were dehydrated in alcohol, cleared in xylol and mounted in Clarite; ten were mounted in glycerine jelly directly following fixation; and 20 were treated according to Laidlaw's modification (1932*a*) of Bloch's (1917) "Dopa"[3] reaction. These preparations were finally mounted in balsam. All scales were studied by both transmitted and reflected light and photographic records made. Appropriate control preparations were reserved for each condition. The ventral surface was carefully examined for pigmentation with a dissecting microscope before each animal was sacrificed and daily observations were made during longer experiments.

Experimental

In connection with studies previously reported (Osborn, 1940*a*), it was observed that experimentally developed melanophores appeared in random positions and patterns on the ventral surface. Pigmented spots of macroscopic size and irregular in shape, differing in intensity from gray to black, appeared here and there over any part of the ventral surface. The only position where melanophores developed with considerable regularity was at the base of the tail. In this area normal control fishes also usually possess some pigment, probably because considerable light reaches this narrow region where the surface is somewhat rounded and unprotected by fins.

The "Dopa" Reaction

This reaction first described by Bloch (1917) and later modified by Laidlaw (1932*a*) has been used for the identification of melanoblasts. Although these cells contain no melanin pigment, Bloch observed the formation of a black substance which he called "dopa-melanin" when treated with "Dopa." He believed this was due to an oxidizing ferment, dopa-oxidase, in the cell which reacted with the "Dopa." Thus, many investigators have interpreted the "Dopa-positive" cell as a potential melanophore even though it had not yet differentiated.

In order to test ventral scales for the presence of melanoblasts by the "Dopa" technique, three groups of flounders (12 fishes in each group) were chosen. The first group was black-adapted, then totally blinded and finally illuminated ventrally to insure optimum conditions

[3] Throughout this paper the term "Dopa" will be used to refer to 3–4 dihydroxyphenylalanin (levorotatory).

for rapid growth of melanophores (Osborn, 1939, 1940*a*). This treatment was continued until considerable ventral pigment had developed (Fig. 6). When a random sampling of ventral scales from such a fish was studied it became apparent that all degrees of pigmentation (melanogenesis) were represented by the various scales (Figs. 8, 9, and 10). In some scales there were no melanophores, in others the small number of melanophores had only scattered melanin granules while still others were melaninated so heavily as to be indistinguishable from scales plucked from the dorsal surface. Such scales arranged in a progressive series show all stages in the acquisition of a full complement of melanin in melanophores, suggesting that the process of pigmentation occurs in the cells *in situ* as they differentiate on a particular scale. Furthermore, there appears to be no tendency for scales adjacent to naturally pigmented areas (the edges of the fins etc.) to become pigmented first with subsequent spreading from originally pigmented surfaces. Rather, melanophores suddenly appear containing a few pigment granules quite independently of neighboring cells. In an attempt to obtain more than circumstantial evidence on this point some scales possessing no melanin-containing cells (microscopic examination—Fig. 2) and others containing but few young melanophores (the exact number and their position on the scale recorded in each instance) were subjected to the "Dopa" treatment. An average of 14 out of 20 scales from each of the 12 fishes gave a positive "Dopa" test (Fig. 3).[4] In some ventral scales positive cells were as numerous as the melanophores on dorsal scales while in other instances only scattered cells responded positively. An entirely satisfactory explanation for the failure of some scales to react positively cannot be given. Two possibilities are suggested: either these scales possessed no melanoblasts, as is apparently the case in scales occasionally found on the dorsal surface, or the technique may not be entirely dependable even though precautions were taken that the solutions were fresh and the incubation temperature accurately controlled.

The flounders in group 2 were illuminated ventrally for a shorter period (4 to 10 days), only until the first appearance (macroscopic) of partially pigmented scales here and there over the surface. For the "Dopa" test scales were chosen which possessed no melanin-containing cells or but few melanophores (again carefully recorded). In this group an average of 16 out of 20 scales per animal responded positively. The range of variation was wide as in the first group.

[4] It is of interest to note that these "Dopa" positive cells appear similar to the round melanoblasts pictured by Laidlaw (1932*b*; Fig. 5, Plate 84; and Fig. 8, Plate 85) in human skin.

The third set of flounders was not subjected to ventral illumination or any other laboratory conditions. They were used immediately without allowing time for adaptation to any unnatural background. Adequate scales were plucked from the ventral surface for each of the three types of preparations previously listed and routine "Dopa" tests were run. None of the cells of the scales used possessed microscopically detectable melanin granules. An average of 13 out of 20 scales from each fish reacted positively to "Dopa." Again the range of variation in the number of positive cells from scale to scale was wide. It is of considerable interest, however, that flounders taken directly from nature should possess numerous potential melanophores on a surface so free from melanin.

Observations Using Transmitted Light

A brief summary follows for the microscopic observations of ventral scales studied by transmitted light. Some were mounted in glycerine jelly to preserve the alcohol-soluble pigments; others were mounted in Clarite following dehydration and xylol clearing.

In glycerine mounts the numerous leucophores appear slightly opaque (Fig. 2) because of their content of guanin crystals and may be easily recognized by their relative numbers, irregular (dendritic) shape and their size. Other cells, less numerous, flattened, and smoother in contour, almost round (in fact having the same shape as those which reacted positively to "Dopa"), could be seen scattered among the leucophores. They are believed to be melanoblasts and are best seen when the iris diaphragm is partly closed. Young melanophores, containing few melanin granules, are of much the same appearance but usually are given a slightly more irregular form by the extensions of simple processes. In studying scales arranged in series progressing from those having no melanophores to scales possessing numerous melanin-containing cells there appears to be a direct correlation between the increase of melanin contained in the cell and the complexity of the processes. A coincidence observed so regularly that it should not be overlooked was that wherever several melanophores were growing in a group the absence of leucophores in that spot was strikingly obvious (Figs. 9 and 10). Viewed with reflected light this was even more easily seen. This suggests that in some way a substitution of melanophores for leucophores may take place or that conditions in the tissues favoring the generation of new melanophores may also be responsible for the degeneration of leucophores. Can it be that leucophores change into melanophores? The very existence on the dorsal surface of structures which apparently contain both melanin and reflecting material (probably guanin) is evidence supporting the idea that two pigments may occur within a single cell (melanoleucophore—Figs. 4 and 5).

It was noted also that in scales possessing many melanophores the cells appeared to be larger and more complex with more numerous, irregular processes in contrast with other scales which had perhaps a half dozen or less melanophores usually of uniform small size and simple pattern, apparently less highly differentiated. To a certain extent the melanophores on a particular scale tend to differentiate more or less synchronously. The way in which experimental pigmentation first appears on the ventral surface of the flounder seems to be in harmony with this and with the evidence gained in the "Dopa" tests. In addition, occasional cells containing some yellow pigment (xanthophores) were seen.

Clarite mounts showed essentially the same picture except that no xanthophores were detected. The leucophores were much more transparent but could be recognized by reducing the light. The smaller round cells were also visible.

Observations with Reflected Light

Glycerine mounts viewed with reflected light showed the leucophores in clear relief but to the disadvantage of the other cells present. However, in cases where some melanophores had developed among the leucophores, the negative outline of the melanin-containing cells could be followed, aided somewhat by the absence of leucophores at that site (see previous page and Figs. 9 and 10). Now and then xanthophores were observed by reflected light.

The scales cleared and mounted in Clarite were less instructive when viewed with reflected light. Because their relative transparency reduced the clarity of the reflected image, they supplied little additional information.

Observations Concerning Regenerating Scales

In areas of injury on the ventral surface where scales had been scraped away, the newly regenerated ones appeared darkly melaninated if the fish was maintained in a physiological and experimental condition favorable to the development of ventral pigment. Such scales are black with melanophores as they appear (Fig. 7). However, if the injured flounders are white-adapted or on a pale natural background with normally alternating night and day (not excessive illumination), the regenerating ventral scales will be white with leucophores and possess no

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Explanation of Figures

Fig. 1. White ventral surface of a freshly caught summer flounder. Note that the scales are normally covered with leucophores (containing guanin) but that melanophores fail to develop on this surface. About ⅙ natural size.

Fig. 2. Photomicrograph of a scale (mounted in glycerine) plucked from the ventral surface of a normal summer flounder. This scale possessed no melanophores. The numerous gray-appearing cells are leucophores which appear slightly opaque when photographed with transmitted light. About 20 ×.

Fig. 3. Photomicrograph of a scale which reacted positively to the "Dopa" treatment. The densely opaque cells which have deposited dopa-melanin are interpreted to be melanoblasts. Before treatment this scale appeared similar to that in Fig. 2. About 20 ×.

Fig. 4. Photomicrograph (*transmitted* light) of a small area of the tip of a scale plucked from the center of a white "excitation spot" on the dorsal surface of a black-adapted flounder. The melanophores are numerous even in this white area but are only slightly dispersed and well concealed by guanin crystals, as will be seen in Fig. 5 taken with reflected light. Cell "x" is the same structure marked for purposes of orientation in Figs. 4 and 5. About 100 ×.

Fig. 5. The same area as shown in Fig. 4. This photograph was made with *reflected* light, however, Note that the total area appears relatively white as it would on the fish in reflected light even though the scale is heavily melaninated. The reflecting guanin appears to be within the bounds of the melanophores because the cells retain a constant size and shape when viewed by reflected and transmitted light. Such a structure is referred to as a "melanoleucophore." Compare Figs. 4 and 5 cell for cell. About 100 ×.

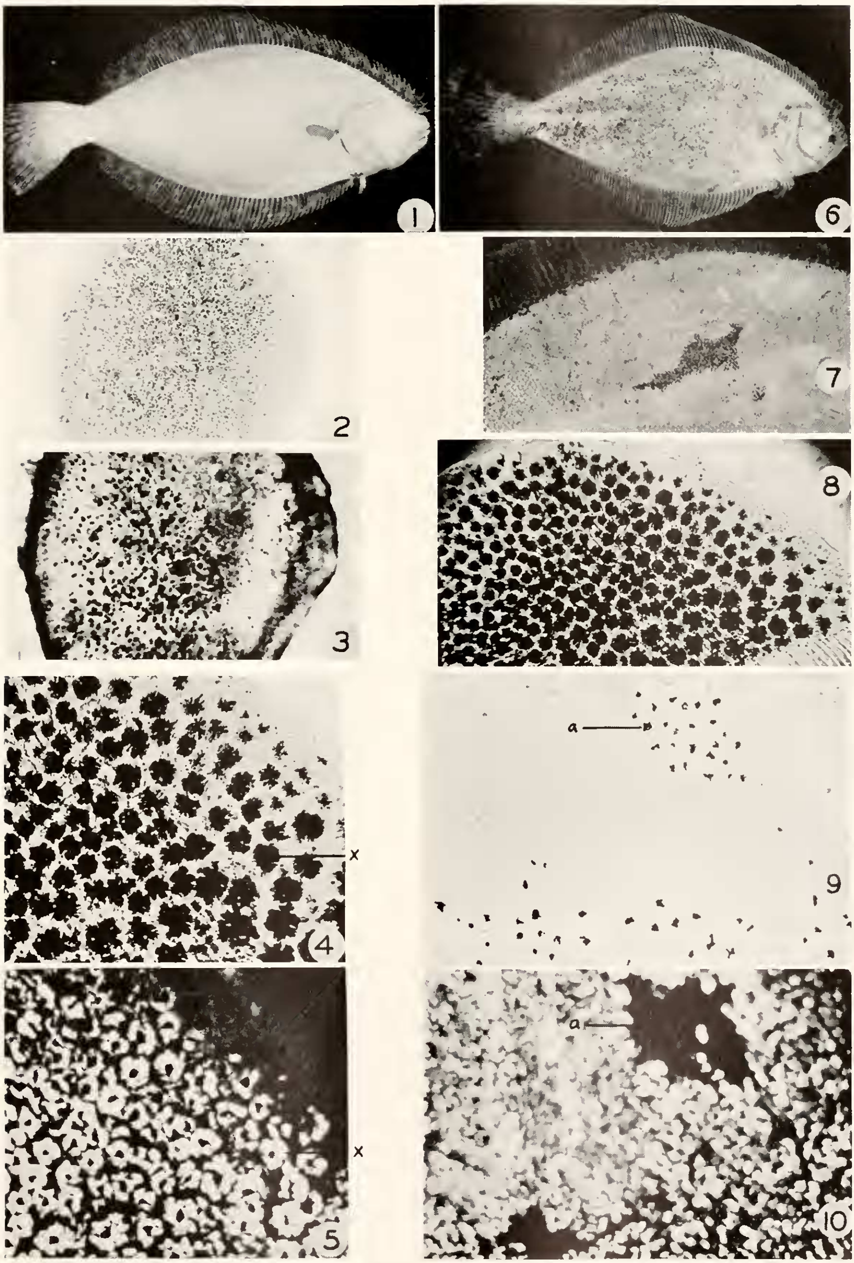
Fig. 6. Ventral view of summer flounder blinded immediately after capture and continuously illuminated ventrally (direct light). Although this fish was illuminated only 18 days, its melanination is nearly as extensive as on the flounder shown in Fig. 7. This is due to the greater efficiency of direct illumination. One-sixth natural size.

Fig. 7. An area of ventral surface adjacent to the pectoral fin. The flounder was black-adapted, blinded, and illuminated continuously 74 days in a white tank. Widespread melanophore formation has occurred but pigmentation is blackest where regenerated scales have grown in an injured area from which the scales had been scraped. One-third natural size.

Fig. 8. Photomicrograph of the tip of a dorsal scale plucked from one of the white "excitation spots" of a black-adapted flounder. The numerous melanophores are only slightly distended during excitation despite the fact that the fish had been black-adapted several days. Scales possessing comparable melanination are commonly found in the darker pigmented areas ventrally. About 50 ×.

Fig. 9. Photomicrograph of part of a scale plucked from a lightly melaninated area of the ventral surface of a summer flounder black-adapted 7 days, blinded and placed in a white tank constantly illuminated from overhead for 12 days. The photograph was taken with *transmitted* light. The few young melanophores present appear in distinct contrast to the numerous leucophores which cover nearly the entire scale surface. Note that the leucophores are absent from the newly melaninated area. Melanophore "a" serves as a point of reference and orientation in Figs. 9 and 10. About 40 ×.

Fig. 10. Same area shown in Fig. 9. Photographed with *reflected* light, the numerous leucophores appear white as on the lower surface of the normal flounder. They contain no melanin and are true leucophores. The melanophores are visible only because they reflect the least light and appear black in contrast with the rest of the scale surface. Compare Figs. 9 and 10. About 40 ×.



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melanophores. On the other hand, if the dark flounders are injured, then placed immediately in the apparatus providing ventral illumination, the scales which regenerate appear as some of the darkest on the entire ventral surface. Although the reason for this is not definitely known, it may be that the numerous leucophores on the white scales tend to mask the developing melanophores during their early stages. The prompt appearance of melanin-containing cells in areas of repair far removed from normally pigmented regions lends further support to the *in situ* origin of ventral melanophores and makes the possibility of migration from previously pigmented areas seem very improbable.

Discussion

That melanophores may be grown under proper experimental conditions on surfaces naturally white and non-melaninated is now well established. The conditions favoring such growth are also known (Osborn, 1939, 1940*a*, *b*, and *c*) although many details await further investigations. The experimental requirements are two-fold: (1) That the surface in question must receive light either directly or by reflection and (2) that the physiological condition (nervous and hormonal factors) of the fish must be such that the internal environment of the normally pigmented cells favors dispersion [5] of the melanin granules (physiological darkening). Odiorne (1937) concluded that this condition also favored the slower morphological darkening in *Fundulus*. Similar findings have been reported in the lower vertebrates by Vilter (1931), Sumner and Wells (1933), Sumner and Fox (1935), Sumner and Doudoroff (1937), Sumner (1939, 1940*a* and *b*), Osborn (1939 and 1940*c*), and Dawes (1941).

The possible source of experimentally developed melanophores attracted the attention of Cunningham (1893), who saw no pigment migrating from the upper surface and so from negative evidence concluded the cells developed *in situ*.

The positive evidence presented here although partly circumstantial supports the view that these melanophores develop where they are first observed. The evidence gained from the direct observation (using both transmitted and reflected light) of small cells whose appearance and distribution agree in general with the picture seen in positive "Dopa" preparations requires no further comment. Likewise the observations concerned with the melanination of regenerated scales strongly favor

[5] That the degree of dispersion need not be maximal is shown by the fact that considerable ventral pigment may be grown on an animal whose dorsal surface is intermediate in shade. It is important to emphasize that the fish should not be in the pale phase.

the *in situ* origin of these melanophores. Furthermore, the fact that evidence gained in using the " Dopa " reaction as an indicator fits well into the other findings suggests that in this instance the reaction is significant and reliable. It is fully appreciated, however, that a positive " Dopa " reaction because of its non-specificity may in itself be of limited value or may prove to be misleading unless supported by evidence from other sources. In these experiments the " Dopa " reaction was used only to test for the presence of positive cells, thus avoiding the necessity of interpreting the implications of the chemical reaction. Because this problem is highly controversial at present some further discussion may be appropriate. That the positive " Dopa " test need not necessarily indicate the presence of a specific oxidizing enzyme (" dopa " oxidase) in the cell has been suggested by Figge (1940), who found that " Dopa " would react with other substances under test tube conditions to produce a black deposit. However, a characteristic feature of a melanoblast is that it contains some substance which will cause " Dopa " to react, forming a black material. This may be formed by a substance which oxidizes the " Dopa " or which acts as a redox substance to accelerate the auto-oxidation of " Dopa " in the absence of an enzyme. This latter possibility seems rather unlikely.

Another possibility is that the cells believed to be melanoblasts on the ventral scales may contain tyrosinase which for some reason has failed to react with tyrosine to produce pigmentation. Figge (1940) suggested that a positive " Dopa " reaction might indicate the presence in a cell of tyrosinase whose oxidizing action was inhibited by a glutathione-like substance. Such substances are known to inhibit the action of tyrosinase on tyrosine but do not inhibit the action of tyrosinase on " Dopa " (Figge, 1940). Tyrosinase actually blackens " Dopa " faster than tyrosine. It is seen then, that the observations made can be explained on a theoretical basis, although we do not, of course, know precisely what happens in the cell.

The question may be asked: " If the melanoblasts are present on the ventral surface, why do they not finish differentiation normally by manufacturing pigment? " It has been demonstrated that they do differentiate to true melanin-containing melanophores when the proper conditions are supplied. One might argue that the internal environment is the same in the ventral cells as in the dorsal cells and that the external environment differs only with regard to the amount of light which normally reaches the upper and lower surfaces of the flounder respectively. If this assumption is true, the following speculation may be offered. Is it possible that in the potential melanophores of the ventral scales a tyrosine-tyrosinase reaction is inhibited by a glutathione-like reducing agent in the absence of light and that exposure to light (experimentally) might remove the inhibiting effect of the reducing agent and allow the enzyme to oxidize the color substrate? That the above assumption is not entirely true, however, is suggested by other observations. The internal environments for the cells of the dorsal and ventral scales are presumably alike in their hormonal constituents but not necessarily so in regard to their respective innervations. This is not known. Pouchet (1876) suggested, however, that a partial atrophy of the sympathetic system may accompany the migration of the corresponding eye during metamorphosis. In view of the present findings concerning ventral pigmentation, further experiments designed to provide new information on the possibility of the degeneration of sympathetic fibers to the ventral surface are needed.

Summary

Melanophores differentiate on the normally non-melaninated ventral surface of summer flounders when two conditions are satisfied. (1) The surface must be exposed to some light source when (2) the animal is in a physiological condition favoring darkening as witnessed by the behavior of the dorsal melanophores.

The melanophores develop "*in situ*" from potential melanophores (melanoblasts) whose presence is evidenced by the positive "Dopa" reaction, by direct observations of various stages of differentiation using direct and reflected light, by studies on regenerating scales, and by additional physiological data.

Theoretical considerations of the possible reactions involved in the experimental development of ventral melanophores and speculations as to why they are normally absent from the ventral surface are presented.

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